PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of

Applicant(s)	:	Catherine M. Verfaillie et al.
Application No.	:	10/561,826
Filed	:	October 17, 2006
Title	:	Neuronal Differentiation of Stem Cells
Examiner	:	Chang Yu Wang
Art Unit	:	1649
Attorney Docket	:	890003-2006.1

DECLARATION UNDER UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Sir:

The undersigned, Catherine M. Verfaillie, Ph.D., declares and states:

I am Professor of Medicine and Director Stem cell Institute, KULeuven, Leuven I am a co-inventor on the above-captioned patent application.

I am the subject of the attached *Curriculum Vitae* and author of the publications listed on the attachment to the *Curriculum Vitae*. On the information and facts contained in those documents, I submit that I am an expert in the field of Stem cell research. In view of these credentials, I believe that I am qualified to speak on the skill and knowledge of the person of ordinary skill in these fields.

I have read and understand the subject matter of the above-captioned patent application. I have read the first Office Action, dated March 17, 2008, and the second Office Action, dated December 24, 2008. I have read and understand the references cited by the Examiner to support the rejections in the Office Actions. These include U.S. 2003/0211605 to Lee et al. ("Lee") and WO 02/086073 to Studer et al. ("Studer").

It is my opinion, based on the scientific evidence and reasoning below, that the rejections are based on an incorrect assumption about (1) how cells should respond to basic fibroblast growth factor (bFGF), fibroblast growth factor 8 (FGF8), Sonic Hedgehog (SHH), and brain-derived neurotropic factor (BDNF) when these are administered simultaneously as opposed to when these are administered sequentially, and (2) the cell type being acted upon by bFGF, FGF8, SHH, and BDNF in the cited art as opposed to the claimed method.

In the earlier Office Action, on page 6, the Examiner rejects the claims as being obvious over Studer in view of Lee. In the later Office Action, the Examiner provides particular reasons for this rejection. Specifically, the Examiner appears to believe that one would generally expect the same result whether one adds all factors simultaneously or sequentially. Specifically, the Examiner states on page 7 of the Office Action, "...at the end of the final steps, the culture medium contains the identical growth factors as those in Studer's to induce neuronal differentiation." The Examiner then reasons, "...because at the end the culture medium still contains the same growth factors and the same cultured ES cells, ...would be induced to differentiate into neurons." (Emphasis added.) Essentially, the Examiner appears to take the position that sequential exposure should have no significant effect because the factors and the cells exposed to them are the same. I do not agree with this reasoning for the reasons that follow.

The End Product of Sequential Exposure to Factors Cannot be Reasonably Predicted Based on the Results of Simultaneous Exposure to the Same Factors

When one introduces a cocktail of factors, as Studer and Lee have done, all the factors are exposed at once to one discrete cell type as to functional, transcriptional, translational, and morphological characteristics. Thus, the factors are acting at the same time on the same cell. Such exposure produces a certain end product. But, in the claimed methods, there are three phenotypically discrete cell types that are being acted upon: (1) the starting cells, (2) the cells that have been exposed to bFGF but not to FGF8 and SHH, and (3) the cells that have been exposed to bFGF, FGF8 and SHH, but not BDNF. Each mitogen would have a specific effect on the cell to produce a phenotypically discrete cell type. So, if one

exposes a cell to bFGF, FGF8, SHH, and BDNF at the same time, the FGF8/SHH is not acting on a cell with a phenotype created by exposure only to bFGF; and the BDNF is not acting on a cell with a phenotype created by exposure only to bFGF, SHH, and FGF8. Therefore, contrary to what the Examiner asserts, when all the factors are in the medium, they are not acting on the "same cultured ES cells." Because of this, one cannot reasonably predict that the result will be the same as the result obtained by Studer and/or Lee.

Although we have not done an experimental comparison between the Studer and Lee end products and the end products obtained using our own steps a) through c), I will discuss an application of the principle illustrated with a differentiation protocol that we conducted in our laboratory pertaining to differentiation of adult bone marrow stem cells into functional hepatocyte-like cells (Snykers et al., *Toxicological Sciences*, 94:330-341 (2006)). Although the reference is directed to differentiation of stem cells into hepatocytes, the principle applies: sequential exposure to factors can result in quite a different end product than simultaneous exposure ("cocktail").

As reported in this reference, we compared the end product obtained by using a cocktail of factors versus sequential exposure to the factors. I will not go into great detail about this reference as the Examiner is fully qualified to assess the reference. However, I will briefly give an outline of the rationale and the results. It had been previously been shown that bone marrow stem cells could differentiate into hepatocyte-like cells from a simultaneous exposure to a mixture of cytokines and growth factors. To try to improve the end product, the cells were exposed to the same factors in a sequential way. Characterization of the cells over a period of time and after exposure to each factor were characterized in several ways: (1) morphology; (2) mRNA expression of hepatocyte-specific genes; (3) protein expression of hepatocyte-specific genes; and (4) hepatic functionality as assessed by albumin secretion, ureogenesis, glycogen storage, and CYP protein expression activity and inducibility.

Our results showed the following. With respect to morphology, using the sequential procedure, the stem cells acquired morphological features similar to those of primary hepatocytes, particularly polygonal-

shaped and bi-nucleated cells. In contrast, using the previous approach, a heterogenous population of epithelioid cells and other cell types was obtained with no polygonal-shaped cells and only a few bi-nucleated cells. With respect to liver associated genes and proteins, more than 85% of these epithelioid cells expressed these genes and expressed them in a comparable time-dependent manner as observed during *in vivo* liver embryogenesis. In contrast, with the cells exposed to the cocktail, the expression patterns differed from the normal sequence in that HNF1α expression preceded that of albumin. In addition, significantly lower levels of liver-specific markers were expressed. With respect to functional maturation, this occurred with both experimental protocols but to a different extent. Hepatic metabolic functions, including albumin secretion, urea production, etc., were manifested most prominently upon sequential exposure to hepatogenic factors.

This illustrates the principle that the end product of sequential exposure to factors cannot be reasonably predicted based on the results of simultaneous exposure to the same factors.

My understanding is that the rejection of obviousness must be based on motivation to change the Studer/Lee procedure. It is my opinion that one would not have been motivated to alter the procedure of Studer and/or Lee because they would not have reasonably expected to produce the same result. Further, my understanding is that the rejection of obviousness must include a reasonable expectation that the same end product would be obtained. As I understand it, another way of looking at this is that it must be reasonably predictable that the same end product would be produced by simultaneous and sequential exposure to the factors. The Examiner seems to assert that position. But, it is my opinion as an expert in the field, that it was not reasonably predictable that the end product in the prior art would be produced by sequential exposure to the factors.

Summary

In the Studer and Lee method, one discrete cell type (neuronal commitment) is exposed to all three factors at once to create a discrete, functional, morphological, and transcriptional and translational profile. Using

the sequential method, however, the starting cells are first exposed to bFGF, which creates a cell with a specific morphology, function, and transcriptional and translational profile. It is this cell that is acted upon by SHH and FGF8, not the original starting cell as in the "cocktail" method. Then, exposure to FGF8 and SHH produces a cell with a second discrete type of morphology, function, and transcription and translational profile. It is this cell that is acted on by the BDNF, not the original starting cell as in the "cocktail" method. That is what makes the end product not reasonably predictable.

Studer/Lee Do Not Apply the Factors to Multipotent Cells

I also point out that Studer/Lee do not apply bFGF to embryonic stem cells, i.e., to a multipotent stem cell. Studer/Lee apply bFGF to a cell already committed to a neural fate. Studer/Lee form embryoid bodies from embryonic stem cells and grow these embryoid bodies without any differentiation factors, selecting for the cells that have undergone neural commitment. It is those cells that have undergone neural commitment that are exposed to the mitogen(s), SHH, and FGF8. The person of ordinary skill would have expected that neural commitment was needed prior to the application of mitogen(s) SHH and FGF8.

In contrast, in the claimed methods, bFGF is applied directly to multipotent stem cells and not to a neurally committed cell. In fact, it is the bFGF that induces neural commitment in these cells.

For this reason alone, I believe that the claimed method would not motivate one to practice the claimed method.

CONCLUSION

It is my opinion, based on the scientific evidence and reasoning set forth above, that the rejection lacks sufficient scientific basis for finding obviousness.

In my opinion, the person of ordinary skill in this field would not have been motivated to drastically change the approach of Studer and/or Lee from a cocktail approach to a sequential approach and would

not have had a reasonable expectation that the same results would be obtained if they did change the approach.

The Studer/Lee method applied to a neural committed cell, not an embryonic stem cell; therefore, the person of ordinary skill could not have reasonably expected successful application of the Studer/Lee method (i.e., exposure to mitogen(s) FGF8 and SHH) unless embryonic stem cells were committed to a neural fate before the factors were applied.

All statements made herein of my own knowledge are true and all statements made on information believed to be true. These statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

6/1/09
Date

Catherine M. Verfaillie, Ph.D.

11459190.1

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CITIZENSHIP: Belgium
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EDUCATION: M.D.: U. of Leuven Medical School, Leuven, Belgium, 1975-1982

POST-GRADUATE TRAINING:
Internship: 1982-83, AZ St Jan Hospital, Brugge, Belgium
Residency: 1983-85, U. of Leuven, Belgium
Fellow in Hematology: 1985-87, U. of Leuven, Belgium
Post-doctoral Fellow: 1987-89, U. of Minnesota, Minneapolis, MN

PROFESSIONAL APPOINTMENTS:
Instructor, Dep. of Medicine, University of Minnesota, 1989-90,
Assistant Professor of Medicine, University of Minnesota 1991-1995
Associate Professor of Medicine, University of Minnesota 1995-1998
Professor of Medicine, University of Minnesota 1998-
Director Stem Cell Biology Program, University of Minnesota, 1996-1999
Director, Stem Cell Institute, University of Minnesota, 1999-2006
Buitengewoon Hoogleraar, Katholieke Universiteit, Leuven, 2005-
Director, Stamcel Instituut, Katholieke Universiteit, Leuven, 2005-

Member, BME Graduate Program, since 1992
Member, MICaB Graduate Program, since 1992
Member, MD/Ph.D Graduate Program, since 1994
Member Cancer Center, 1994
Member, GCD Graduate Program, since 2000
Member, Clinical Laboratory Science Graduate Program, since 2000
Member, Neuroscience Graduate Program, since 2002
Member Doctoral School, Department of molecular and Cellular Therapy, K.U.Leuven

CERTIFICATION:
FMGEMS-1987
FLEX-1990

HONORS:
M.D., Summa Cum Laude, 1982
Special Fellow, Leukemia Society of America, 1991
Special Fellow, 'Fundacion Internacional Jose Carreras Para La Lucha Contra La Leucemia ',1991
Young Investigator Award, International Society of Exp. Hematology, 1992
Scholar, Leukemia Society of America, 1995
Outstanding Investigator Award, Central Society, 1996
Elected, Member, American Society of Clinical Investigation, 1996
Tulloch Chair in Stem Cell Biology, Genetics and Genomics, 1999
Anderson Chair in Stem Cell Biology, 1999
Elected, Councilor, American Society of Clinical Investigation, 2001
McKnight's Presidential Chair in Stem Cell Biology, 2001
Elected, Member, American Association of Professors, 2003

Vice President, International Society of Experimental Hematology, 2002

President Elect, International Society of Experimental Hematology, 2002
President, International Society of Experimental Hematology, 2004

4th Annual Landazuri Award, University of Navarra, Pamplona, Spain 2002
Damashek Medal, American Society of Hematology, 2002
Honorary Doctorate, Katholieke Universiteit, Belgium, 2003
Distinguished Woman Scholar Award, University Of Minnesota, 2003
2003 Jose Carreras Award, European Society of Hematology, 2003
Forum Engelberg Prize, Lucerne, Switzerland, 2003
Gulden Spoor voor Vlaamse Internationale Uitstraling, Vlaanderen-Europa, 2003
Star Award, Minnesota Hematology Oncology, 2004
Stewart-Niewiarowski Award for Women in Vascular Biology, 2004
Jimenez Diaz Price for Scientific Achievements in Research, 2004
Vlerick Award, 2005
Honorary Member, BeWiSe, 2005
Gabriella Moortgat Prijs, 2006
Bijzonder Hoogleraar, TEFAF Oncology Wisselleerstoel, 2009, Faculty of Health, Medicine, and Life Sciences, Universiteit van Maastricht
Cariplo Professor, University of Pavia, Italy, 2009-2011

NAMED LECTURES:

Presidential Symposium, American Society of Hematology, San Francisco, 2000
Nobel-Forum Lecture series, Karolinska Institute, Stockholm, Sweden, 2000
Linda Laubenstein Memorial Lecture, NYU, New York, 2000
Clement Finch Visiting Professor, University of Washington, Seattle, 2002
4th Annual Landazuri Lecture, University of Navarra, Pamplona, Spain, 2002
Gerhard Smith Memorial Lectureship, City of Hope, CA, 2002
Fiftieth Chalmers J Lyons Memorial Lectureship, AAOMS, Chicago, IL, 2002
Forum Engelberg Award Seminar, Lucerne, Switzerland, 2003
Hohenberg Lecture, university of Pennsylvania, PA, 2003
Kilo Professorship, Washington University, St. Louis, MO, 2003
Evans Lecture, Boston University, Boston, MA, 2003
Stewart-Niewiarowski lecture, Temple University, Boston, MA, 2004
KeKuLe Lecture 2004, Antwerp, Belgium, 2004
NIH Director's Lecture, Washington, DC, 2004
Bendit Lecture, University of Washington, Seattle, WA, 2004
Jimenez Diaz Commemorative Lecture, Madrid, Spain, 2004
Presidential Symposium, ASGT, Minneapolis, MN, 2004
Latta Lecture, University of Nebraska, Omaha, 2005
Presidential Symposium, Am Soc for Investigative Pathology, San Diego, 2005
Moloney Lecture, Brigham & Women's Hospital, Boston MA, 2005
Ada Comstock Inaugural Lecture, Minneapolis, MN, 2005
Brecher Lecture, San Francisco, CA, 2005
Visiting Professor Department of Medicine, Vanderbilt University, 2006
Presidential Symposium, ESTRO, Leipzig, Germany, 2006
Pierre Stryckmans Memorial Lecture, Brussels 2007

PROFESSIONAL ASSOCIATIONS:

American Federation for Clinical Research (AFCR)
American Society of Gene Therapy (ASGT)
American Society of Hematology (ASH)
American Society of Stem Cell and Bone Marrow Transplantation (ASBMT)
International Society for Hematotherapy and Graft Engineering (ISHAGE)
International Society of Experimental Hematology (ISEH)
International Bone Marrow Transplantation Registry (IBMTR)
Autologous Bone Marrow Transplantation Registry (ABMTR)
International Society for Stem Cell Research (ISSCR)

COMMITTEE ASSIGNMENTS (Extramural):

Treasurer, International Society of Experimental Hematology, 1997-2001
Councilor, American Society of Clinical Investigation, 2001-2005
Board of Directors, American Society of Blood and Bone Marrow Transplantation, 2001-2005

Councilor, Society Cell transplantation, 2002-2006
Member, Scientific Committee of the European School of Hematology, 2004-
Chair, Policy Committee, ISSCR, 2002-2006
Member, Scientific Subcommittee on Transfusion Medicine, ASH, 1996-1998
Member, Scientific Subcommittee on Growth Factors, ASH, 1998-2002
Member, Awards Committee, ASH, 2003-2007
Member, Publications Committee, ISEH, 2001-2005
Member, Committee on Hemopoietic Cell and Gene Therapy, ASGT, 98-02
Member, Stem Cell Evaluation Committee, ISHAGE, 1997-2001
Member, Mesenchymal Stem Cell Committee, ISHAGE, 1999-2004
Member, Stem cell Expansion Committee, ISHAGE, 2001-2003

Chair, VA Merit Award Heme Study Section, 2001-2002
Member, NIH study section, Heme I, 2000-2004
Member, VA Merit Award Heme Study Section, 1999-2002
Member, LSA Translational Awards Review Committee, 1999-2004
Member, Telethon Scientific Committee, Italy, 2001-2005
CIRM review committee, 2006-
Review committee, Connecticut Stem Cell Initiative, 2006-
Member CNRS review panel, Belgium, 2008-2012

Ad hoc reviewer, Juvenile Diabetes Research Fund
Ad hoc reviewer, Muscular Dystrophy Association
Ad hoc reviewer, Leukemia Research Fund, Great Britain
Ad hoc reviewer, Welcome Trust, Great Britain
Ad hoc reviewer, Research Council, Canada
Ad hoc reviewer, Nationaal Fonds Wetenschapelijk Onderzoek, Belgium
Ad hoc reviewer, Associazone Italiana per la Ricerca Sul Cancro
Ad hoc reviewer, Israel Science Foundation
Ad hoc reviewer, Dutch Cancer Society
Ad Hoc reviewer, Medical and Health Services Research Division, Ireland.
Ad hoc reviewer, European Commission, FP6
Ad hoc reviewer, European Commission, FP7
Ad hoc reviewer, European Research Council, 2007-

COMMITTEE ASSIGNMENTS (Intramural):
Promotion and Tenure Committee, Department of Medicine 1997-2001
Research Committee, Department of Medicine, since 1997
Steering Committee MD/Ph.D. Program, 1998-2005
Molecular Medicine Planning Committee, 1998-2000
AHC, Functional Genomics Advisory Group, 2000-2004
Consortium on Law and Values in Health, Environment & the Life Sciences, 2001-6

CONSULTANT/ADVISOR
Member International Scientific Advisory Board, UK Government and Wellcome Trust Joint Infrastructure Fund, 1999
Member Advisory Committee, Institute of Hematology, Chinese Academy of Sciences & Peking Union Medical College, 2000-2005
Member, Advisory Committee Tissues of Life Project, Science Museum of Minnesota,2000-2006
Member Scientific Advisory Panel, University of Nebraska Stem Cell Biology Research Center, 1999-2004
Member Scientific Advisory Panel for National Stem Cell Resource, Corriel Institute, Camden, NJ, 2000-2004
Member, Advisory Committee, Mayo Clinic Myeloma PO1, 1998-2004
Member, Stem Cell Advisory Committee, National Research Institute, Taiwan, 2002-
Consultant, Athersys Inc., Cleveland, OH, 2002-
Member, Advisory Board, Center for Transgene Therapy and Gene Therapy, VIB, Leuven, Belgium, 2003
Member Advisory Panel, Stem Cell GAP, NIH, 2003-5
Member, Scientific Advisory Board, Oncostem Therapeutics, Salamanca, Spain, 2004-2007
Member, Scientific Advisory Board, Toronto McLaughlin Centre, 2004-2007
Member, Scientific Advisory Board, Framework 6 Program Beta Cells, 2004
Member, Scientific Advisory Board, DPTE, 2005-
Member, Scientific Advisory Board, Case Western University Stem Cell Institute, 2005-2008
Lid Raad van Advies, EOS, 2005-

Member Advisory Committee, Itinera, 2006-
Advisory Board Regenerative Medicine, the Netherlands, 2007-2009
Co-Chair Research and Quality Assurance Evaluation, Lund University, 2008
Member, Review Committee Science Foundation Ireland REMEDI CSET, 2008
Member, Scientific Advisory Committee, Fondazione Roma, 2008-
Member, Advisory Board, FP7 Infarct Cell Therapy project, PI E Hofer, 2008-
Member International Advisory Board Norway Stem Cell Center, 2008-
Member Advisory Board, EC project "Infarct Cell Therapy", 2009-

ASSOCIATE EDITOR:

Experimental Hematology, 2003-2008
Experimental Hematology, 1998-200
Leukemia, 1997-2002
Hematologia, Citocinas, Immunoterapia Y Terapia Cellular, 1997-2002
Stem Cell Reviews, 2004-2008
PloS-1, 2008-

EDITORIAL BOARD:

Blood, 1995-1999
Experimental Hematology, 1996-1998
Leukemia, 1996-1997
Cytotherapy, 1999-2003
Journal of Biology of Blood and Marrow Transplantation, 2001-2005
Cloning & Stem Cells, 2001-2005
Current Gene Therapy, 2005-
Journal of Engineering and Regenerative Medicine, 2006-
Stem Cells, 2007-2008
Stem Cells International, 2008-

PATENTS:

WO9718298: Ex vivo culture of stem cells
CA2381292: Multipotent adult stem cells and methods for isolation
US2007022482: High-throughput functional analysis of gene expression
WO2006086639: Vascular/lymphatic endothelial cells
WO2006047743: Swine multipotent adult progenitor cells
WO2005045012: Endodermal stem cells in liver and methods for isolation thereof
WO2005003320: Neuronal differentiation of stem cells
WO2008063675: Endodermal progenitor cells
US2002081733: Method to prepare drug-resistant, non-malignant hematopoietic cells
WO2004050859: Homologous recombination in multipotent adult progenitor cells
WO9513088A1: Stroma-derived stem cell growth factors
WO9320184: Method for culturing hematopoietic cells
WO2002040718: Method to identify genes associated with chronic myelogenous leukemia
AU2006304318: Differentiation of non-embryonic stem cells to cells having a pancreatic phenotype
AU2005331534: Use of MAPC or progeny therefrom to populate lymphohematopoietic tissues
US11/808933: High Oct3/4 MAPCs and methods therefor
US 61/022121: Stem cell aggregates and methods for making and using
US08/82108: Optimized methods for differentiation of cells into cells with hepatocyte and hepatocyte progenitor phenotypes, cells produced by the methods, and methods for using the cells
US - GB 0822483.4: Maintenance/expansion of HSCs
US 60/690089 *: HSC Self-Renewal (CIP of US2007022482)

TRAINEES:

High School students		**Current Position**
Evan Cobbs	2002-2003	U of Madison, undergraduate
Nicole Ali	2002-2004	Harvard University, undergraduate
Ricky Jones	2002-2004	CalTech, undergraduate
Sam Bjork	2003-2005	Harvard University, undergraduate

Undergraduate Students		**Current Position**
Venita Chandra	1997-1998	Medical School, U of Chicago

Sarah Aldrich	2000, 2001	Medical School, U of Chicago
Aaron Lisberg	2001, 2002	MD/PhD program,
Zach Kastenberg	2002-2004	Medical School, U. of Minnesota
Eric Rarhman	2002-2005	Graduate School, U of Minnesota
Lee Sandquist	2003-2005	Medical School, U of Minnesota
Juliana Hagenbrock	2003-2006	PhD Student, University of Amsterdam, The Netherlands
April Breyer	2003-2006	Law School, Boston College Law School
Jennifer Gravelle	2003-2005	Medical School, U of Minnesota
Mike Felten	2003-2005	Medical School, U of Minnesota
Thomas Szynski	2003-2005	Medical School, Harvard U

Medical Student Advisor		**Current Position**
Sjoban Keel, B.S.	1997-1998	Fellow, Hematology, U. of Washington
Troy Lund, Ph.D.	1998-2002	Pediatric Heme-Onc Fellow, U of Minnesota
David Dyle, B.S.	1998-2000	Fellow, Genetics, U. of Washington
Zubaid Rafique, B.S.	2003-2005	
Tzu-Fei Wang. M.S.	2004-2005	
Eleanor Chen, Ph.D	2004-2006	Pathology Residency, Harvard U

Medical Resident Research		**Current Position**
Jade Anderson, M.D.	1999-2000	Fellow, Hematology, U. of Minnesota
Ken Lee, M.D.	2000-2001	Fellow, Cardiology, UCSF

Medical Resident Advisor		**Current Position**
Sjoban Keel, M.D.	2000-2001	Fellow, Hematology, U. of Washington
David Dyle, M.D.	2000-2004	Fellow, Genetics, U. of Washington

Masters Students		**Current Position**
Yuehua Jiang, M.D.	1997-1999	Assist. Prof., U of Minnesota
Sofia Melikova, B.S.	2000-1002	Scientist, R&D Systems, Minneapolis
Ben Vaessen, B.S.	2000-2002	Scientist, Glaxo-Smith-Kline
Avinash Jayaswal, B.S.	2002-2003	Medical School, UCL, Brussels, Belgium
Qing Cai	2006-2007	PhD Student, K.U.Leuven
Janick Beckers	2006-2007	PhD Student, K.U.Leuven
David Zwaenepoel	2006-2007	Scientist, ReGenesys, BVBA
Adriaan Campo	2006-2007	PhD Student, U. Antwerpen
Maria Aelberts	2007	PhD Student, U. of Copenhagen
Antonio LoNigro	2007-2008	PhD Student, K.U.Leuven
Simone Calzolari	2007-2008	PhD Student, U. of Barcelona
Kim Van Uytsel	2007-2008	PhD Student, K.U.Leuven
Jasper Wouters	2007-2008	PhD Student, K.U.Leuven
Tine Verryckt	2007-2008	
Olivier Govaere	2007-2008	PhD Student, K.U.Leuven
Lotte Vanbrabant	2007-2008	Technician, K.U.Leuven
Vijay Kumar	2008	PhD Student, U of Toronto
Alessandra Familiari	2008-2009	
Caterina DiPrieto	2008-2009	

Graduate Students:		**Current Position**
Beverly Lundell, MS.	1993-1996	Scientist, Aastrom, Michigan
Eugene Liu, M.D.	1996-2000	Assist Prof of Medicine, U. of Taipei, Taiwan
Scott Dylla, M.S.	1998-2002	Post-doc, Irving Weissman, Stanford U
Fernando Ulloa, B.S	2002-2006	Scientist, Glaxo Smith Kline
Lucas Chase, B.S.	2003-2006	Scientist, Invitrogen
Eric Mendenhall, B.S.	2003-2006	Post-doc, Bernstein lab, MIT
Lepeng Zeng, B.S.	2003-2006	Scientist, Medtronic
Jeff Ross, B.S., M.S.	2003-2006	Scientist, Surmodics
Ben Kidder, B.S.	2003-2007	Postdoc, Serono, Boston
Shannon Buckley, B.S.	2004-2009	
Annelies Crabbe, BS	2006-	
Valerie Roobroeck, BS	2006-	

Kartik Subramanian, BS	2006-	
Yonsil Park, BS	2006-	
Jason Owens, BS	2007-	
Qing Cai, BS, MS	2007-	
Kim Van Uytsel, BS, MS	2008-	
Antonio LoNigro, BS, MS	2008-	
Rojin Abraham MD	2008-	
Elda Mineola, MD	2008-	

MD/PhD students:		**Current Position**
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Robert Schwartz, B.S.	2000-2004	Fellow Gastroenterology, Harvard University, Boston
Craig Eckfeldt, B.S.	2002-2005	Resident Internal Med, U. of Minnesota
Sarah Frommer, B.S.	2002-2006	Medical School, U of Minnesota
Terri Burns, B.S.	2003-2007	Medical School, U. of Minnesota

Postdoctoral *Fellows*

Chunjin Ding, M.D.	1992-1994	Scientist, Ely Lilly, Indianapolis
Huilin Qi, Ph.D.	1998-2003	Scientist, Yale University
Dean Aguiar, Ph.D.	1999-2001	Staff Scientist, Pharmacia
Yuehua Jiang, MS, MD	1999-2002	Assist. Prof., U of Minnesota
Stephanie Salesse, Ph.D.	2000-2005	Assist. Prof, U of Reims, France
Mo Dao, Ph.D.	2001-2004	Post Doc, N Taylor, Montpellier, France
Troy Lund, MD, Ph.D.	2002-2003	Assist. Prof, Department of Pediatrics, U of Minnesota
Yves Heremans, Ph.D.	2002-2005	Instructor VUB, Belgium
Uma Lakshmipathy, Ph.D.	2002-2005	Staff Scientist, Invitrogen, CA
Beatrice Pelacho, Ph.D.	2002-2005	Instructor, U of Navarra, Pamplona, Spain
Aernout Luttun, Ph.D.	2002-2006	Assist. Prof, K.U.Leuven, Belgium
Miguel Barajas, Ph.D.	2004-2006	Assist. Prof., U of Navarra, Pamplona, Spain
Marta Serafini, Ph.D.	2004-2006	Assist. Prof., Fondazione M.Tettamanti M.De Marchi Onlus, Monza, Italy
Rik Snoeckx, Ph.D.	2005-2009	Postdoctoral fellow, J Cools lab, VIB-KULeuven
Kris Van den Boogaert, Ph.D.	2005-	
Carlos Clavel, Ph.D.	2005-2008	Postdoc, Einstein University, USA
Fernando Ulloa, Ph.D.	2006-2008	Scientist, Glaxo Smith Kline
Martine Geeraerts, Ph.D.	2006-	
Pau Sancho-Bru, Ph.D.	2007-2009	
Takeshi Shimizu, Ph.D.	2007-	
Jeroen DeClercq, Ph.D.	2007-	
Bipasha Bose, Ph.D.	2008-2009	
Anujit Kumar, Ph.D.	2008-	
Satish Kumar, Ph.D.	2009-	
Yong Li, Ph.D.	2008-	

Fellows		**Current Position**
Jeffrey S Miller, M.D.	1991-1994	Prof. of Medicine, Director, Translational Research Program, Cancer center, U. of MN
Pankaj Gupta, M.D.	1992-1995	Prof. of Medicine, U. of MN
Randolph Hurley, M.D.	1992-1995	Staff Physician, Health Partners, St Paul, MN
Ravi Bhatia, M.D.	1992-1996	Prof. of Medicine, Director Stem Cell Program, City of Hope
K.Y. Chiang, M.D., Ph.D.	1993-1995	Assist Prof. Pediatrics, Emory University, Atlanta GA
Vivek Roy, M.D.	1994-1996	Assist. Prof. of Medicine, Mayo Clinic, FL
Robert Zhao, M.D., Ph.D	1995-1998	Professor, Professor, Chinese Acad. of Sci. & Peking Union Medical College
Michael Punzel, M.D.	1996-1998	Assoc Prof., U. of Duesseldorf, Germany
Felipe Prosper, M.D.	1995-1997	Prof. of Medicine and Director Stem Cell Program, U. of Navarra, Pamplona, Spain
Michel Delforge, M.D.	1995-1996	Assoc. Prof, KULeuven, Belgium
Juliet Barker, M.D.	1997-1999	Assoc. Prof. of Medicine, Sloan Kettering Institute, NYC
Ian Lewis, M.D., Ph.D.	1997-1999	Assoc. Prof. of Medicine, U. of Adelaide, Australia
Chris Lamming, M.D.	1999-2002	Pediatric Fellow, Baylor College, Houston, TX
Claudio Brunstein, M.D.	1999-2002	Assist Prof. of Medicine, U of Minnesota

Koen Theunissen, M.D.	1999-2001	Hematologist, Virga Jesse Hospital, Hasselt
Balkrishna Jahagirdar, M.D.	2000-2002	Assist Prof. of Medicine, U of Minnesota
Eugene Liu, M.D., Ph.D.	2000-2002	Assist Prof. of Medicine, U. of Taipei, Taiwan
Mojca Jongen, M.D.	2001-2003	Instructor Hematology, U. of Rotterdam, NL
Anskar Leung, M.D., Ph.D.	2001-2003	Assist. Prof, U of Honk-Kong
Masayuki Oki, M.D.	2003-2006	Assist. Prof, Tokai U, Tokio, Japan
Catherine Flynn, M.D.	2004-2007	Consultant Haematologist, St James's Hospital and Coombe Women's Hospital, Dublin
Karen Pauwelyn, M.D.	2005-2009	Fellow, Hepatology, K.U.Leuven
Helene Schoemans, M.D.	2006-2008	Fellow, Hematology, K.U.Leuven
Philip Roelandt, M.D.	2007-	

INVITED LECTURES (Since 2007)

2007

Keynote Speaker, Basel Switzerland
First Connecticut International Stem Cell Symposium, Hartford, CT
Grand Challenge Meeting 4, Lugano, Switzerland
Symposium on tissue reconstruction, UCL, Brussels, Belgium
8th Advanced Summer Course in Cell - Materials Interactions, Instituto de Engenharia Biomédica, Porto, Portugal
MSC2007, Adult Mesenchymal Stem Cells in Regenerative Medicine, Cleveland, OH
The Second UK Mesenchymal Stem Cell Meeting, U of York, Great Britain
International Symposium "Stem cells, Development and Regulation" Amsterdam, The Netherlands
Symposium on Cardiovascular Regenerative Medicine, NIH, Washington DC, USA
Spanish National Congress of Surgery, San Sebastian, Spain
Scottish Stem Cell Network, Glasgow, Scotland
Interhospital Endocrine Rounds, Montreal, Canada
Research Seminar, Buck Institute, CA
Research Seminar, U Liege, Belgium
Research Seminar, VUB, Brussel

2008

Keynote Lecture, EPISTEM conference, Gent, Belgium
Keynote Lecture, VIB, Blankenberge, Belgium
Keynote Lecture, Italian Society for Biotechnology and Medical Engineering, Rieti, Italy
Keynote Lecture, Wadden Symposium on Diabetes, Texel, the Netherlands
Keynote Lecture, Annual Norwegian Stem Cell Network, Oslo Norway
Keynote Lecture, Itera Conference, Maastricht, the Netherlands
Keynote Lecture, Annual science day of the GROW, Maastricht, The Netherlands
EuroSTELLS, Stem Cell Niche Meeting, Barcelona, Spain
Symposium "Pluripotency and differentiation in embryos and stem cells", Pavia, Italy
The Adult Stem/Progenitor Cell Niche, Brussels, Belgium
Nederlandse Vereniging voor Hematologie, Papendal, the Netherlands
Annual Wound Healing Society Meeting, San Diego, USA
International Stem Cell Meeting, Tel Aviv, Israel
EAE/ESH 2nd symposium on MSC, Mandelieu, France
Summer School, Barsinghause, Germany
Nobel Forum Cancer Stem Cell Conference, Stockholm, Sweden
4th International Conference on Regenerative Hepatology, Dusseldorf, Germany
European Society of Gene Therapy, Brugge, Belgium
Mayo Clinic Stem Cell and Regeneration Symposium, Rochester, MN, USA
The 5th Dubai International Conference for Medical Sciences, Dubai
EU-EPC Roundtable, Brussels Belgium

2009

Plenary Lecture, Annual Meeting, French Society of Blood Transfusion, Strasbourg, France
Plenary Lecture, Annual Meeting , ESGT, Hannover, Germany
The Sanquin Spring Seminar, Amsterdam, the Netherlands
Flanders Bio Seminar Tissue Engineering, Brussels
Research Seminar, UCL, Brussels, Belgium
Research Seminar, U Frankfurt, Frankfurt, Germany
Epiplastcarcinoma Marie Curie RTN network meeting, Leuven, Belgium

MEETING ORGANIZER

Yearly Meeting ISEH, 2005, Glasgow, Scotland
Yearly meeting ISEH, 2006, Minneapolis, MN
Keystone meeting, Stem Cells, 2006, Whistler, Canada
Mesenchymal Stem Cells (1), 2006, Mandelieu, France
Mesenchymal Stem Cells (2), 2008, Mandelieu, France
Muscular Dystrophy Symposium Leuven, October 2, 2008
Stem Cells: Biology and Applications; Sponsored by FP6-STROKEMAP and TEFAF, Leuven, 2009

RESEARCH INTERESTS:

1. NORMAL HEMATOPOIESIS:

- Regulation of normal human hematopoietic stem cell proliferation, differentiation and lineage commitment by cytokines and components of the extracellular matrix using *in vitro* as well as *in vivo* xenogeneic transplant models.
- Molecular characterization of hematopoietic stem cells by functional genomics, and zebrafish model of hematopoiesis

2. PLURIPOTENT STEM CELLS (MAPC, ESC, iPS).

- Purification, expansion and characterization of differentiation to mesodermal, ectodermal and endodermal lineages
- Characterization of molecular determinants of pluripotent and multipotent stem cell phenotype, and of de-differentiation and differentiation
- Evaluation of therapeutic potential in congenital disorders or for the treatment of vascular, neurodegenerative disorders, hepatic disorders and diabetes.

CURRENT FUNDING:

- **PO1-CA-65493-06** (PI P McGlave): Biology and Transplantation of Human Stem cells Project Period: 7/1/2000 - 6/30/2005. Project Leader: project 1; Annual Direct Cost: $210,000/year
- **FWO** (PI Verfaillie) Het potentieel van multipotente adulte progenitor cellen in de vervanging van insuline-secreterende ß-cellen in preklinische modellen van type 1 diabetes. Period 1/1/2007-12/31/2009; annual direct cost: €30,000
- **KUL CoE** (PI Verfaillie) Period: 11/1/2005 - 10/31/2009. Annual Direct Cost: €500,000/year
- **Odysseus Fund** (PI Verfaillie) Period: 12/21/2006 - 12/31/2010. Annual Direct Cost: €1,390,000/year
- **FP6-STREP: STROKEMAP** (PI Verfaillie). Multipotent Adult Progenitor Cells to treat Stroke; Period: 10/1/2006 - 9/30/2009. Total Cost: €2,400,000; Total cost Verfaillie €420,000
- **FP6-STREP: CHRYSTAL,** (partner 6: Verfaillie) Cryobanking of stem cells for human clinical application. Period: 1/1/2007 - 12/30/2009. Total cost Verfaillie: €321,000
- **SBO BRAINSTIM** (PI Verfaillie); non-invasive imaging of stem cells in the brain; Period 10/1/2007-9/31/2011; Total Cost: €2,650,000; Total cost Verfaillie €550,000
- **SBO: IMAGINE** (Partner Verfaillie). Generation of improved paramagnetic particles for stem cell labeling and application in tumor therapy. Period: 1/1/2009 - 12/310/2013. Total cost Verfaillie: €250,000

PENDING FUNDING:

- **FP7: BELISTEM** (Partner Verfaillie). Stem cells suitable for liver regeneration. From the bench to the bed side; Period: 1/1/2010 - 12/30/2014. Total cost partner Verfaillie: €1,100,000
- **FP7: STEMPER** (Partner Verfaillie). Stem cells suitable for therapy of peripheral vascular disease. Period: 1/1/2010 - 12/30/2014. Total cost KULeuven: €1,300,000
- **SBO: HEPSTEM** (PI Verfaillie). Generation of mature hepatocytes from human induced pluripotency stem cells Period: 1/1/2010 - 12/310/2014. Total cost €2,500,000
- **NIH GRANT 10121990** (PI Ekker, Co-I Verfaillie). Genomic Analysis of Hematopoietic Stem Cell Niche Formation, Maintenance & Function. Period: 1/7/2009 – 30/6/2014. Yearly Budget Verfaillie: $125,000
- **Dutch Diabetes Fund** (PI P Devos, Co-I Verfaillie). Human fetal and adult progenitor cells as a source for insulin producing cells. 4/1/20o9 - 31/3/2012. Total cost Verfaillie €250,000

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3. Rummens J, Verfaillie C. Capnocytophaga infections: a risk in the immunocompromised host. Acta Clinica Belgica 39: 2-10, 1984. (IF: 0.58)
4. Louwagie A, Criel A, Verfaillie C. Philadelphia chromosome positive T acute lymphoblastic leukemia. Cancer Genet and Cytogenet 16: 297-300, 1985. (IF: 1.57)

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9. Verfaillie CM, McGlave P. Leukemia inhibitory factor (LIF)/human interleukin for DA cells (HILDA) supports the growth of multipotential hematopoietic cells in vitro. Blood 77: 263-270, 1991. (IF: 10.9)
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11. Verfaillie CM, Weisdorf D, Haake R, Hostetter P, Ramsay NKC, McGlave P. Candida infections in bone marrow transplant recipients. Bone Marrow Transplant 8:177-184, 1991. (IF: 2.6)
12. Verfaillie CM, McCarthy JB, McGlave PB. Differentiation of primitive human multipotent hematopoietic progenitors into single lineage clonogenic progenitors is accompanied by alterations in their interaction with fibronectin. J Exp Med 174: 693-703, 1991. (IF: 15.6)
13. Uhlman D, Verfaillie C, Jones RB, Luikaert SD. BCNU treatment of marrow stromal layers reversibly alters hematopoiesis. Brit J Hemat 78:304-309,1991. (IF: 4.1)
14. Verfaillie CM, Miller WJ, Boylan K, McGlave PB. Selection of benign primitive hematopoietic progenitors in chronic myelogenous leukemia on the basis of HLA-DR antigen expression. Blood 79: 1003-1011, 1992. (IF: 10.9)
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Sequential Exposure to Cytokines Reflecting Embryogenesis: The Key for *in vitro* Differentiation of Adult Bone Marrow Stem Cells into Functional Hepatocyte-like Cells

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Differentiation of adult bone marrow stem cells (BMSC) into hepatocyte-like cells is commonly performed by continuous exposure to a cytokines-cocktail. Here, it is shown that the differentiation efficacy *in vitro* can be considerably enhanced by sequential addition of liver-specific factors (fibroblast growth factor-4, hepatocyte growth factor, insulin-transferrin-sodium selenite, and dexamethasone) in a time-dependent order that closely resembles the secretion pattern during *in vivo* liver embryogenesis. Quantitative RT-PCR analysis and immunocytochemistry showed that, upon sequential exposure to liver-specific factors, different stages of hepatocyte differentiation, as seen during liver embryogenesis, can be mimicked. Indeed, expression of the early hepatocyte markers alpha-fetoprotein and hepatocyte nuclear factor (HNF)3β decreased as differentiation progressed, whereas levels of the late liver-specific markers albumin (ALB), cytokeratin (CK)18, and HNF1α were gradually upregulated. In contrast, cocktail treatment did not significantly alter the expression pattern of the hepatic markers. Moreover, sequentially exposed cells featured highly differentiated hepatic functions, including ALB secretion, glycogen storage, urea production, and inducible cytochrome P450–dependent activity, far more efficiently compared to the cocktail condition. In conclusion, sequential induction of the differentiation process, analogous to *in vivo* liver development, is crucial for *in vitro* differentiation of adult rat BMSC into functional hepatocyte-like cells. This model may not only be applicable for *in vitro* studies of endoderm differentiation but it also provides a "virtually unlimited" source of functional hepatocytes, suitable for preclinical pharmacological research and testing, and cell and organ development.

Key Words: **bone marrow stem cells; hepatocytes; sequential differentiation; liver-specific growth factors; liver embryonic development; *in vitro*.**

INTRODUCTION

Drug development is aimed at identifying pharmacologically active drug candidates with a favorable toxicologic profile. The increasing number of safety criteria, imposed on newly designed molecules, leads nowadays to the urgent need of *in vitro* techniques in the industry, developed according to the principle of Russell and Burch. To date, several hepatocyte-based *in vitro* models are available, however, they are not yet accepted into regulations, as they still require better characterization and optimization to reach the validation stage. Most primary hepatocyte cultures are in fact hampered by progressive occurrence of differentiation (De Smet *et al.*, 2001; LeCluyse *et al.*, 1996; Rogiers and Vercruysse, 1993). An alternative approach would be the use of postnatal progenitor/stem cells.

Indeed, until recently, it was believed that tissue-specific stem cells could only differentiate into cells of the tissue of origin. However, a number of recent studies have suggested that adult stem cells may overcome germ lineage restrictions and express molecular characteristics of cells of different tissue origin, which has been termed "plasticity" (Jackson *et al.*, 2001; Krause *et al.*, 2001; Theise *et al.*, 2000; Vourc'h *et al.*, 2004). For example, hematopoietic cells may acquire characteristics of cardiomyocytes, cells of lung, gut, liver, blood vessels, skin, etc. (Jackson *et al.*, 2001; Krause *et al.*, 2001; Theise *et al.*, 2000). This apparent plasticity can at least in some instances be explained by cell fusion (Wang *et al.*, 2003). Other studies have described nonhematopoietic stem cells from bone marrow that are capable of differentiating *in vitro* in cells with mesodermal, ectodermal, and endodermal features (Jiang *et al.*, 2002; Reyes *et al.*, 2001; Yoon *et al.*, 2005). The mechanism through which these cells gain multipotency is not totally understood (Verfaillie, 2000). Multipotent adult progenitor cells, for instance, can be induced to express phenotypic and functional characteristics of hepatocytes; however, the degree of differentiation obtained till now is incomplete (Schwartz *et al.*, 2002).

Therefore, in order to develop an *in vitro* model suitable for pharmaco-toxicological purposes, attempts were made here to

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optimize the differentiation efficiency of nonhematopoietic stem cells from bone marrow into functional hepatocytes.

Liver development is accomplished by a sequential array of biological events. Each step of cell growth and differentiation is tightly regulated by cell autonomous mechanisms and extracellular signals, including cytokines and growth factors. More specifically, during the initial phase of murine liver ontogeny (embryonic days [E] 8–9), fibroblast growth factors (FGFs), derived from adjacent cardiac mesoderm, commend the foregut endoderm to form the liver primordium (Duncan, 2000; Jung *et al.*, 1999). During and after the mid-stage of hepatogenesis, surrounding mesenchymal cells secrete hepatocyte growth factor (HGF) and support as such the fetal hepatocytes (Kinoshita and Miyajima, 2002; Zaret, 2002). Around E11, the fetal liver becomes the major site for hematopoiesis. During this stage, hematopoietic stem cells produce oncostatin M that, in the presence of glucocorticoids, not only promotes fetal hepatic cell differentiation and maturation but also suppresses embryonic hematopoiesis. In contrast, oncostatin M alone fails to induce differentiated liver phenotypes, implying that glucocorticoids are essential triggers for hepatic maturation (Kinoshita and Miyajima, 2002; Schmidt *et al.*, 1995; Zaret, 2002). In rodents, the final step of hepatic differentiation takes place several days after birth. The lack of terminal differentiation of primary hepatocytes in culture evidences that additional signals, probably generated through the extracellular matrix, are necessary (Kinoshita and Miyajima, 2002).

Here, the liver development was taken as exemplar to establish a culture model that more readily supports robust differentiation of bone marrow stem cells (BMSC) to mature hepatocyte-like cells. We compared two experimental setups: (1) BMSC were treated with a cocktail of liver-specific factors (FGF-4, HGF, insulin-transferrin-sodium selenite [ITS], and dexamethasone [Dex]) as previously described (Schwartz *et al.*, 2002) or (2) innovative in this field, BMSC were exposed to a sequence of these compounds in a manner that closely reflects their temporal expression during *in vivo* hepatogenesis (FGF-4, followed by HGF, followed by a combination of HGF, ITS, and Dex).

MATERIALS AND METHODS

Isolation and culture of undifferentiated rat BMSC. BMSC were isolated from male Fisher rats (4–6 weeks old) and cultured as described by Jiang *et al.* (2002). Labware used for expansion of BMSC included Corning 75 and 150 cm^2 tissue culture flasks, polystyrene (both from VWR, Leuven, Belgium). Cell karyotyping, neuroectodermal, and endothelial differentiation were determined as previously described (Jiang *et al.*, 2002, 2003; Reyes *et al.*, 2001). Rats had access to food and water *ad libitum* and were housed according to guidelines from the Institutional Animal Care and Use Committee of the University of Minnesota.

Hepatocyte differentiation. Rat BMSC from 60 population doublings on were used for differentiation into hepatocyte-like cells. BMSC were plated at 21×10^3 cells/cm^2 on 1 mg/ml collagen type I–coated culture plates and dishes (BD Falcon 24-well plate, polystyrene; BD Falcon 35 × 10 mm petri dishes, polystyrene; NUNC F96 microwell plate, black, polystyrene; NUNC F96 microwell plate, clear, polystyrene [all from VWR]) in low-serum expansion medium (Jiang *et al.*, 2002; Reyes *et al.*, 2001). Once cells reached 100% confluence, they were washed with basal medium (Jiang *et al.*, 2003) supplemented with 0.03mM nicotinamide, 0.25mM sodium-pyruvate and 1.623mM glutamine (all from Sigma, Bornem, Belgium). Subsequently, cells were cultured in the presence of liver-specific cytokines and growth factors, added either as a cocktail (basal medium + 10 ng/ml FGF-4, 20 ng/ml HGF [all from R&D Systems, Minneapolis, MN], 1 × ITS and 20 μg/l Dex [all from Sigma]) or sequentially (days 0–3: basal medium + 10 ng/ml FGF-4; days 3–6: basal medium + 20 ng/ml HGF; from day 6 on: basal medium + 20 ng/ml HGF + 1× ITS and 20 μg/l Dex). Differentiation media were changed every 3 days. A schematic presentation of the differentiation procedure is shown in Figure 1.

Quantitative RT-PCR. For PCR analysis, 1 μg RNA was reverse transcribed to cDNA using Superscript II reverse transcriptase and random hexamer primers (Invitrogen, Merelbeke, Belgium). The resulting RT-products were essentially amplified as previously described (Jiang *et al.*, 2003; Schwartz *et al.*, 2002). Three extra steps were included to ensure the purity of the PCR products: 95°C for 15 s, 60°C for 20 s, and 95°C for 15 s. The primers used for amplification and the products expected are described in (Jiang *et al.*, 2003; Schwartz *et al.*, 2002). The RNA levels were normalized using 18S and compared with the RNA levels in undifferentiated BMSC (negative control) and freshly isolated primary rat hepatocytes (positive control). As a negative control for the primers, a no template cDNA-PCR reaction was run under the same conditions. The authenticity and size of the PCR products were confirmed by melting curve analysis (using software provided by Perkin Elmer, Lennik, Belgium) and gel electrophoresis.

Immunocytochemistry. Differentiated BMSC were fixed either with ethanol for 10 min at − 20°C (cytoskeletal proteins) or with 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) for 10 min at 4°C, followed by incubation with 100mM glycin to saturate reactive groups (nuclear and cytoplasmic markers). The fixed cells were permeabilized for

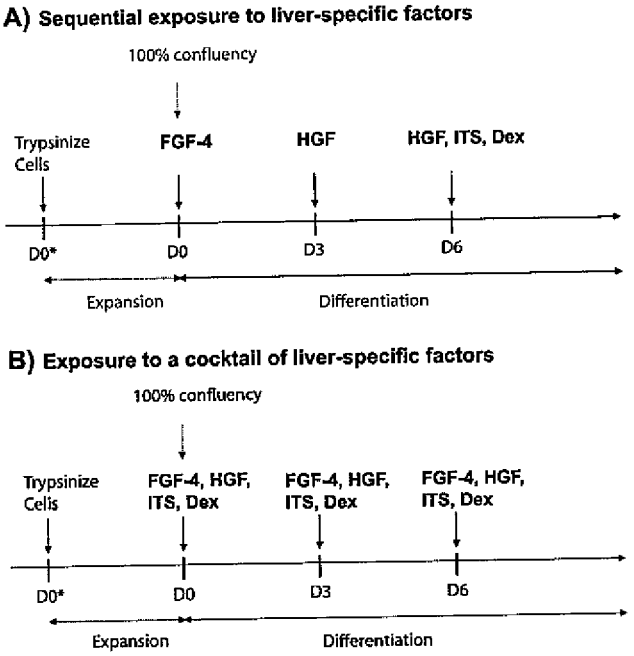

FIG. 1. Schematic presentation of the differentiation protocol. BMSC, at 100% confluency, were exposed either sequentially (A) or simultaneously (B) to liver-specific factors. D0*, day that BMSC were plated at 21×10^3 cells/cm^2 on collagen type I in low-serum expansion medium.

15 min with 0.1% Triton in phosphate-buffered saline (Electron Microscopy Sciences) and blocked for 30 min with 1% bovine serum albumin/5% donkey serum block buffer at room temperature. After blocking, cells were incubated overnight at 4°C with primary antibody (fluorochrome-conjugated or non-conjugated) and washed three times with phosphate-buffered saline. In case the primary antibody was not conjugated, cells were incubated for 2 h at room temperature with secondary fluorochrome-conjugated antibody. After incubation, slides were washed again with 0.1% Triton in phosphate-buffered saline and mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, CA). As a negative control, cells were incubated with appropriate gamma immunoglobulines (Jackson Immunoresearch, Cambridgeshire, UK) and immunostained under the same conditions. In order to evaluate the localization of cytochrome P450 (CYP) proteins, mitochondria and endoplasmic reticulum were counterstained with the carbocyanine dye $DiOC_6$ (Molecular probes, Invitrogen). Cells were analyzed using fluorescence microscopy with a Zeiss Axiovert scope. To enumerate the number of cells expressing a given marker, all nuclei of positive-stained cells were counted and compared to the total number of cells evaluated. The primary antibodies against alpha-fetoprotein (AFP) (goat), hepatocyte nuclear factor (HNF)3β (goat), and HNF1α (rabbit) were purchased from Santa Cruz, (Heidelberg, Germany). Anti-cytokeratin (CK)18 (mouse, FITC-conjugated) and anti-albumin (ALB) (goat, FITC-conjugated) antibodies were from Sigma and Bethyl Laboratories (Montgomery, TX), respectively. The antibodies against CYP1A1 and CYP2B1/2 (both goat) came from Daiichi pure chemicals, BD Biosciences (Tokyo, Japan). Respective secondary antibodies were purchased from Jackson Immunoresearch.

Albumin ELISA. ALB concentrations, secreted into the culture media, were analyzed by ELISA (Koebe *et al.*, 1994).

Urea assay. The produced urea concentrations were, after 24-h exposure of the cells to 6mM NH_4Cl, colometrically measured in culture media according to the manufacturer's instructions (Quantichrom Urea assay kit, Bioassay Systems, Brussels, Belgium). Fresh culture media supplemented with 6mM NH_4Cl and 4 h-cultured adult rat hepatocytes were used as a negative and positive control, respectively.

Glycogen storage. Intracellular glycogen was analyzed by Periodic-acid-Schiff staining (PAS-kit 395B-1KT, Sigma) according to the manufacturer's instructions. Amyloglucosidase (Sigma)-treated cells and 4 h-cultured adult rat hepatocytes were used as a negative and positive control, respectively.

Alkoxyresorufin-O-dealkylase assay. Ethoxyresorufin-O-deethylase (EROD) and pentoxyresorufin-O-dealkylase (PROD) activities were assessed as previously described (Donato *et al.*, 1993) with some minor modifications: in our setup, cells were incubated with 20μM 7-ethoxyresorufin and 18μM 7-pentoxyresorufin (all from Sigma) for 30 min.

To evaluate the inducibility of CYP2B1/2 and CYP1A1/2, respectively, cells were, after 24 days of differentiation, exposed to phenobarbital (PB; final concentration 1mM) and 3-methylcholantrene (MC; final concentration 2μM; all from Sigma). Media, supplemented with either PB or MC, were daily renewed from that time on. Fresh culture media and 4 h-cultured adult rat hepatocytes were used as a negative and positive control, respectively.

Statistics. Results are expressed as mean ± SD. Statistical analyses were performed using one-way ANOVA and Student's *t*-test. The significance level was set at 0.05.

RESULTS

Characterization of the Differentiation Pattern of Rat BMSC into Hepatocyte-like Cells: Sequential versus Cocktail Exposure

Morphological Features

Previously, it has been shown that BMSC could differentiate into hepatocyte-like cells upon simultaneous exposure to a mixture of well-defined cytokines and growth factors (Schwartz *et al.*, 2002). However, using this approach, a rather heterogeneous population of epithelioid cells and other cell types was obtained. Moreover, no polygonal-shaped cells and only few binucleated cells were formed (Fig. 2). In an attempt

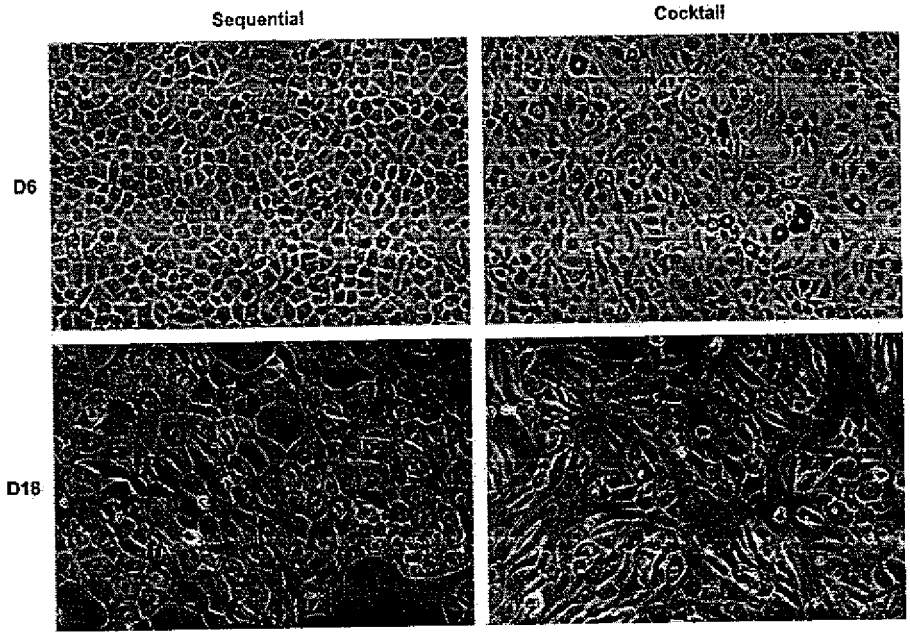

FIG. 2. Light-microscopic analysis of BMSC-derived hepatocyte-like cells upon sequential or simultaneous exposure to liver-specific factors at days 6 and 18. Original magnification of 20 × 10, phase contrast.

to improve the differentiation of nonhaematopoietic stem cells from bone marrow into hepatocyte-like cells, BMSC were exposed to the same well-defined hepatogenic factors, but in a sequential way. More specifically, cytokines and growth factors were added at defined points in time, in a manner that closely resembles the *in vivo* process of embryonic liver development as specified in "Materials and Methods" section (Duncan, 2000; Jung *et al.*, 1999; Kinoshita and Miyajima, 2002; Schmidt *et al.*, 1995; Zaret, 2002). In this novel setup, epithelioid cells appeared in culture from day 6 on (Fig. 2). However, at that moment these cells were still surrounded by spindle-shaped cells. After 14 days, less fibroblastic cells were seen and some binucleated cells appeared. After 18 days, most cells exhibited a polygonal shape (Fig. 2).

Characterization at the Molecular Level

In a next set of experiments, we evaluated whether these morphological differences were associated with distinct patterns of differentiation at the molecular level. Therefore, the expressions of early (AFP and HNF3β) and late (ALB, CK18, and HNF1α) liver-specific markers were analyzed at both the mRNA (Fig. 3) and protein levels (Figs. 4 and 5).

mRNA expression. In both sequential and cocktail culture conditions, AFP, HNF3β, ALB, CK18, and HNF1α were expressed in a time-dependent manner during BMSC differentiation. Both the pattern and the level of expression, however, differed considerably between the culture methods. In fact, upon sequential exposure to liver-specific factors, maximal AFP mRNA expression occurred after 6 days (Fig. 3), 4 days later than seen in the cocktail condition, but was 1.2-fold higher than the maximal level observed in cocktail-exposed cells. AFP mRNA expression disappeared completely in both conditions by day 11 of culture. In sequentially treated cells, downregulation of AFP mRNA expression was nicely followed by a second transient, though more pronounced, induction of the early liver-specific marker HNF3β as well as by a steady upregulation of the late hepatic markers ALB, CK18, and HNF1α (Fig. 3). More specifically, HNF3β mRNA expression started at day 2, reached maximal levels at day 10 and decreased rapidly thereafter (Fig. 3). ALB and CK18 mRNA expression, on the other hand, gradually increased from days 4 and 10 of culture, respectively, until maximal levels were reached at day 18 (Fig. 3). In sharp contrast to these observations, changes in HNF3β, ALB, and CK18 mRNA levels were negligible upon simultaneous exposure to all hepatogenic factors ($p < 0.001$; one-way ANOVA). In addition, the mRNA of the late liver-specific marker ALB remained very low in cocktail-exposed BMSC, suggesting an immature hepatic differentiation status. Finally, upon sequential exposure, HNF1α

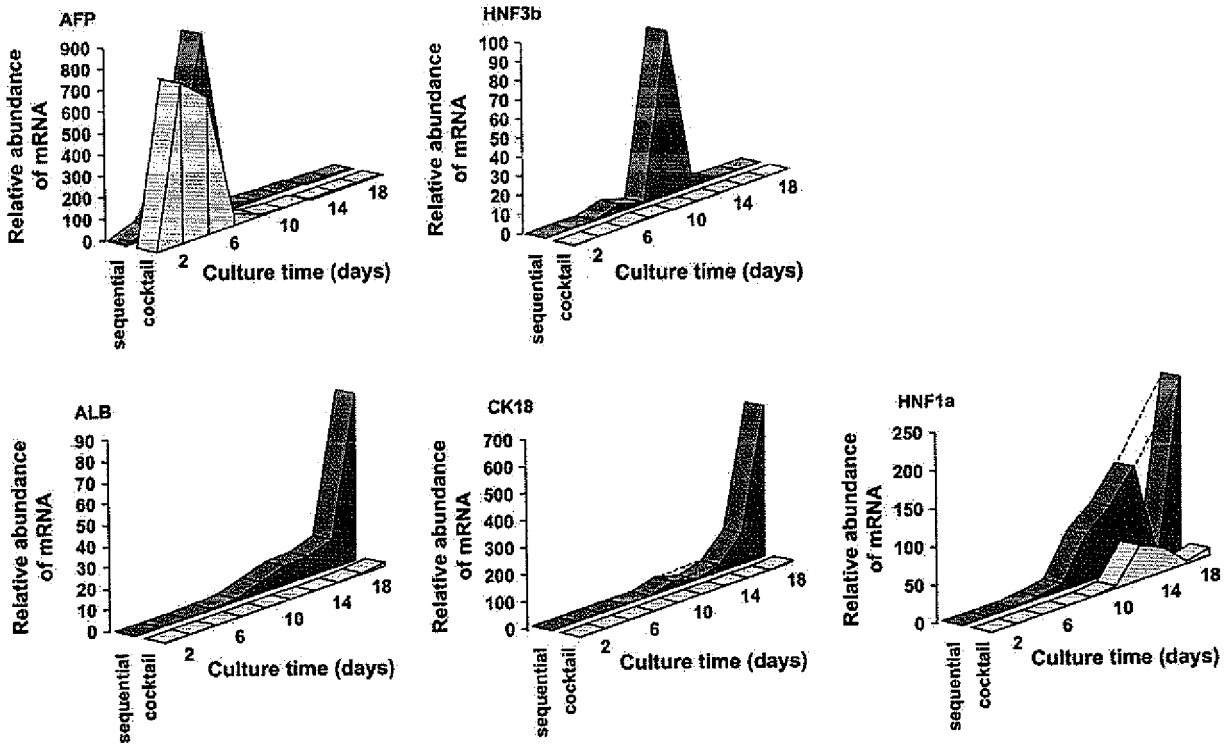

FIG. 3. Analysis of hepatocyte differentiation at the mRNA level (abundance in cultured cells relative to freshly isolated rat hepatocytes [%]). BMSC were either sequentially or simultaneously exposed to liver-specific factors. Values represent means of three independent experiments. The dotted lines represent nontested time points. They are assumptions, based on the existing expression pattern.

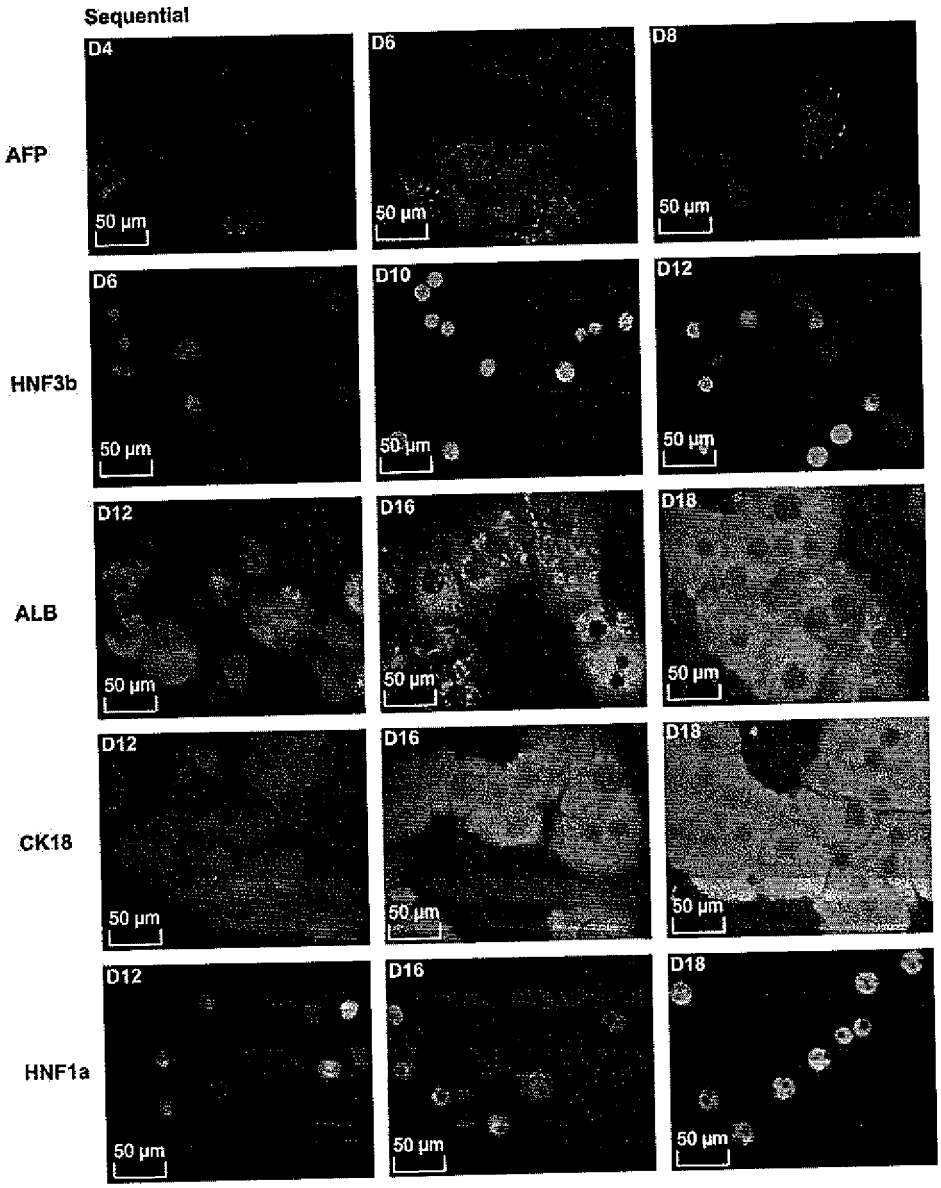

FIG. 4. Characterization at the protein level of BMSC differentiation into hepatocyte-like cells upon sequential exposure to liver-specific factors. Immunocytochemistry was performed for AFP-cy3, HNF3β-cy3, CK18-FITC, HNF1α-cy3, and ALB-FITC. Nuclear counterstaining was assessed using DAPI. Original magnification of ×320. Scale bar, 50 µm. Stainings shown have the same magnification and are representative for at least five separate experiments.

mRNA expression gradually increased from day 6 on whereas in the cocktail condition, HNF1α mRNA induction was delayed by 4 days and occurred only transiently (Fig. 3). Moreover, maximal levels, obtained at day 12, were about twofold lower than the levels observed in 12-day–old BMSC in the sequential condition. Thus, sequentially exposed BMSC underwent a consecutive array of developmental stages comparable with *in vivo* hepatogenesis while exposure to a cocktail of cytokines and growth factors induced an aberrant expression pattern of differentiation when compared to liver embryogenesis.

Protein expression. In order to support the results obtained at the mRNA level, immunocytochemistry analyses were performed in parallel (Figs. 4 and 5). After 4 days of differentiation, cells expressed AFP, regardless of the experimental setup (Figs. 4 and 5). Concomitantly to the results found at the mRNA level (Fig. 3), AFP expression occurred only transiently in both conditions (Figs. 4 and 5) and was undetectable by day 12 of culture (data not shown). Upon sequential exposure to liver-specific factors, a maximal positive staining of HNF3β (92 ± 8%) was noticed at day 10, leveling off thereafter (Fig. 4). Treatment with all factors

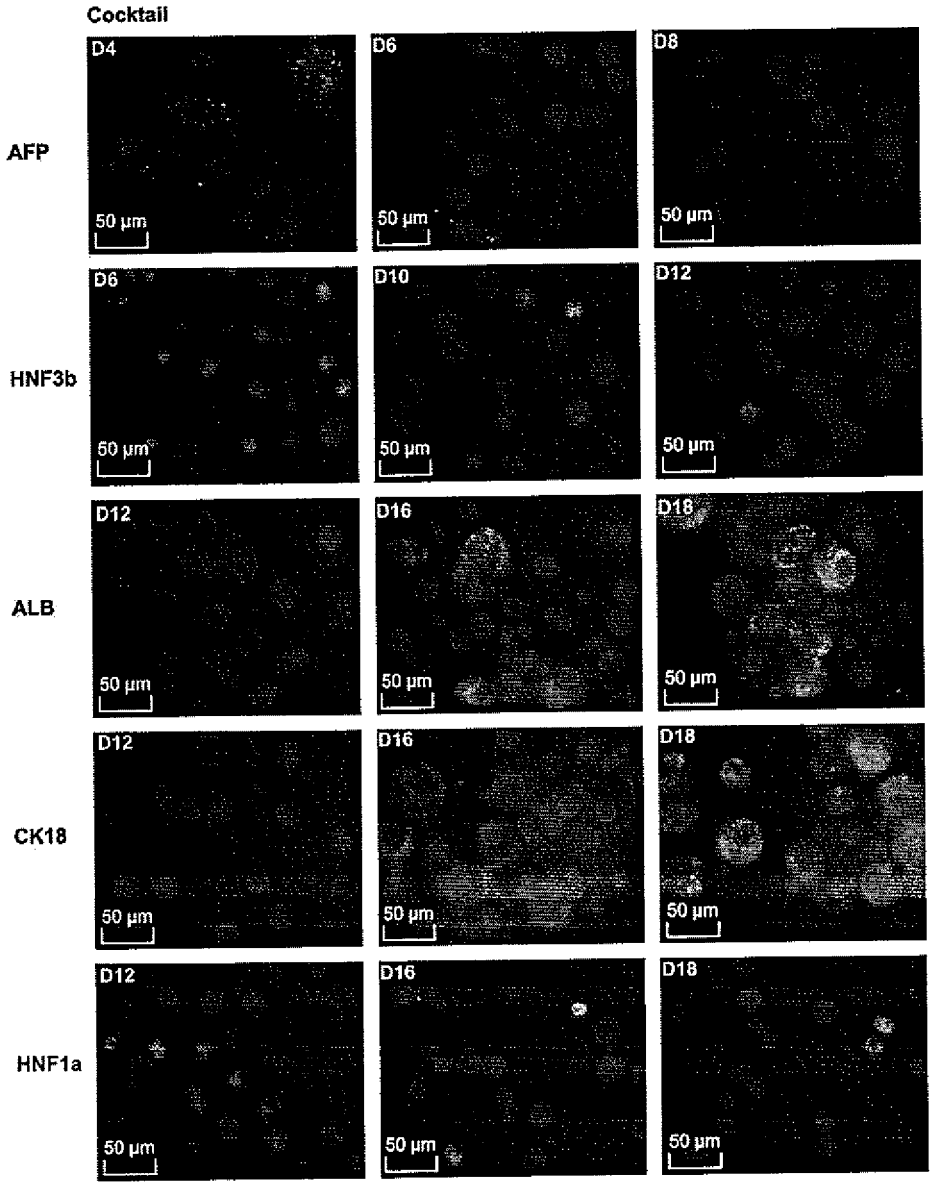

FIG. 5. Characterization at the protein level of BMSC differentiation into hepatocyte-like cells upon simultaneous exposure to liver-specific factors. Immunocytochemistry was performed for AFP-cy3, HNF3β-cy3, CK18-FITC, HNF1α-cy3, and ALB-FITC. Nuclear counterstaining was assessed using DAPI. Original magnification of ×320. Scale bar, 50 µm. Stainings shown have the same magnification and are representative for at least five separate experiments.

simultaneously, however, revealed no more than 24 ± 7% HNF3β-positive cells throughout the culture period (Fig. 5). As differentiation progressed, extensively increased stainings for ALB, CK18, and HNF1α were detected upon sequential exposure to cytokines and growth factors, in accordance with the results obtained at the RNA level (Figs. 3 and 4). Consequently, after 18 days, 92 ± 2%, 94 ± 3%, and 89 ± 9% of the cells, respectively, stained positive for these markers (Fig. 4), which is in sharp contrast to only 32 ± 4%, 63 ± 5%, and 22 ± 4% of the cocktail-exposed cells, respectively ($p < 0.001$; Student's t-test) (Fig. 4).

In addition, in order to state the immunocytochemistry data with certainty, immunoblotting has been performed in parallel once (data not shown). In line with the previous results obtained at both the mRNA and protein level, sequentially exposed cells expressed liver-specific proteins more abundantly than cells in the cocktail setup. However, since this approach consumes large numbers of cells, i.e., at least 25–50 µg of protein is needed to analyze one liver-specific marker at one point in time, the analysis was not repeated. Alternatively, as measuring CYP activity (the set of EROD/PROD) and their inducibility are widely accepted as final end point to evaluate the suitability of

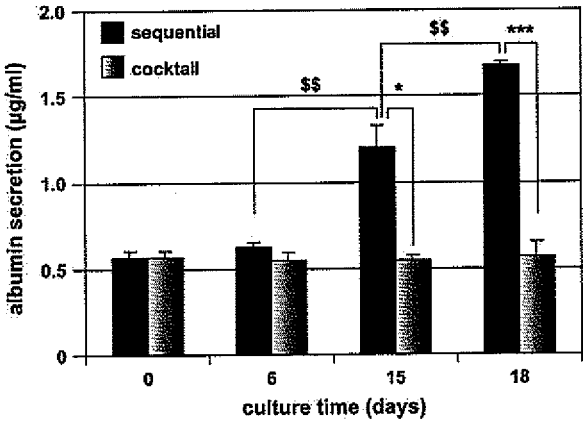

FIG. 6. ALB secretion in sequentially- and cocktail-exposed BMSC. The results shown are representative for five independent experiments, each performed in duplicate. *, ***: ALB-secretion significantly differs among sequentially- and cocktail-exposed BMSC with $p < 0.05$ and $p < 0.001$, respectively (Student's t-test). $$: ALB-secretion in sequentially exposed BMSC is significantly upregulated from day 15 on with $p < 0.01$ (Student's t-test).

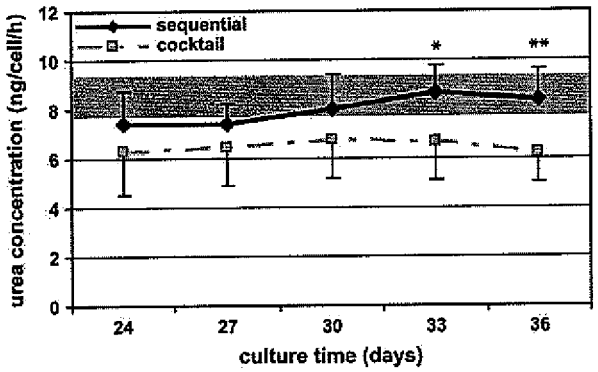

FIG. 7. Urea production in sequentially- and cocktail-exposed BMSC. Gray area represents urea levels, produced by 4 h-cultured adult rat hepatocytes. The graph is representative for four separate experiments, each performed in duplicate. *, **: Urea production significantly differs among sequentially- and cocktail-exposed BMSC with $p < 0.05$ and $p < 0.01$, respectively (Student's t-test).

cells as *in vitro* models for pharmaco-toxicological screening of drugs (De Smet *et al.*, 2001; Donato *et al.*, 1993, 2003; LeCluyse *et al.*, 1996; Rogiers and Vercruysse, 1993), we enlarged, in a next set of experiments, the data set on cell functionality in order to increase confidence in our data.

Hepatic Functionality

In order to assess whether these hepatocyte-like cells derived from the bone marrow also acquired typical functional hepatic features, ALB secretion, ammonia metabolism, glycogen storage, expression of CYP proteins in parallel with their activity and inducibility were evaluated.

ALB secretion. Sequentially treated BMSC significantly upregulated the ALB secretion rate from day 15 onward ($p < 0.01$, Student's t-test) (Fig. 6). On the contrary, BMSC exposed to a cocktail of liver-specific factors did not secrete ALB above basal levels, corresponding to 0.55 µg/ml (Fig. 6).

Ureogenesis. Upon sequential exposure to hepatogenic factors, the urea production increased over culture time, reaching adult levels after 30–33 days. In contrast, cocktail-exposed cells synthesized, even at peak production, 24% significant lower urea levels ($p < 0.05$; Student's t-test) (Fig. 7).

Glycogen storage. Furthermore, upon sequential treatment with cytokines and growth factors, glycogen uptake was first seen after 21 days of culture, 6 days earlier than in the cocktail condition. After 30 days of culture, about 86% of the cells stored glycogen, regardless of the culture method (Fig. 8).

CYP protein-expression, activity, and inducibility. In the sequential setup, phase I CYP1A1 and CYP2B1/2 proteins were expressed within and nearby the endoplasmic reticulum and mitochondria (Fig. 9). The level of expression gradually increased as differentiation progressed. After 30 days, 78 ± 1 and 79 ± 3% of the cells stained positive for CYP1A1 and CYP2B1/2, respectively (Fig. 10). In sharp contrast to these observations, cocktail-exposed cells only showed modest CYP expression over culture time (Figs. 9 and 10).

In addition, we investigated whether CYP1A1 and 2B1/2 were functionally active by measuring the respective EROD and PROD activities in both conditions (Figs. 11 and 12).

In line with the results found at the protein level, sequentially exposed cells exhibited markedly higher EROD and PROD activity rates compared to the cocktail model ($p < 0.05$ at days 36 and 39; Student's t-test) (Figs. 11 and 12). Upon sequential exposure to liver-specific factors, a transient fourfold increase in PROD activity was displayed by days 27– 30, approaching the level of 4 h-cultured adult rat hepatocytes, versus only a twofold increase after cocktail treatment (Fig. 11). In addition, in the former setup, EROD activity gradually increased from days 27 to 36 towards levels measured in 4 h-cultured adult rat hepatocytes, whereas CYP1A1/2-dependent activities appeared only transiently in cocktail-exposed cells between days 30 and 33 and declined to almost nondetectable levels on day 36 (Fig. 12).

CYP-inducibility is considered as the most representative metabolic function of the adult hepatic phenotype (Gomez-Lechon *et al.*, 2004; Rogiers and Vercruysse, 1993). Therefore, the responsiveness of both CYP1A1/2 and CYP2B1/2 to their respective prototype inducers MC and PB was analyzed in parallel. PROD activities were induced up to 1.4-fold after 6-day exposure to PB (i.e., on day 30), regardless of the experimental setup (Fig. 11). The inducibility persisted for 6 days in sequentially exposed cells but not in the cocktail condition. A significant CYP1A1/2-dependent response to MC was observed on days 36–39 ($p < 0.001$ and $p < 0.01$ at days 36 and 39, respectively; Student's t-test) in the sequential model. Conversely, MC barely induced EROD activities upon culture with all liver-specific factors simultaneously (Fig. 12).

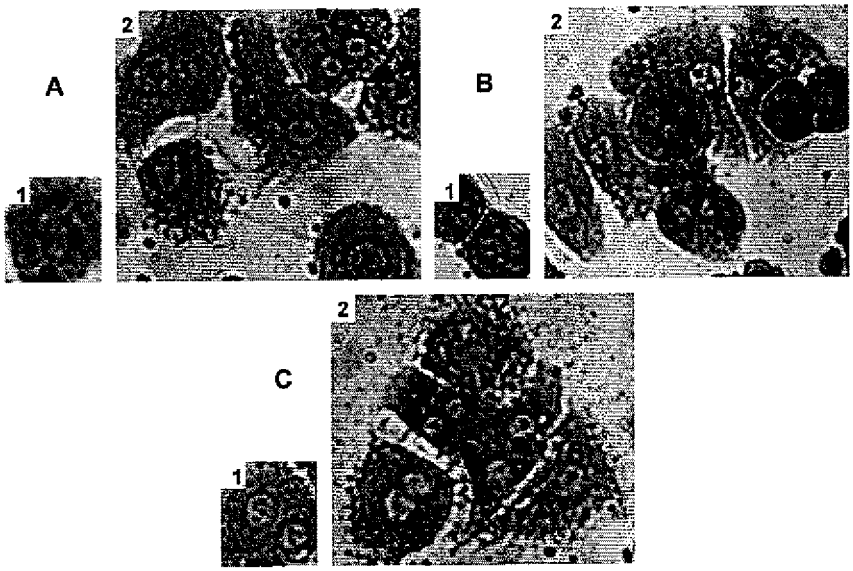

FIG. 8. Glycogen storage in 30-day–old sequentially- (A) and cocktail-exposed BMSC (B) and 4 h-cultured adult rat hepatocytes (C), in the presence (1) and absence (2) of amyloglucosidase, respectively. Glycogen and nuclei are colored magenta and blue, respectively. Original magnification of ×400. Stainings shown are representative for three separate experiments.

DISCUSSION

In recent years, adult-derived stem cells have become a hot topic in the field of molecular, cellular, and clinical biology, as well as in pharmaco-toxicology. Indeed, stem cells have an extensive self-renewing potential and many of them are considered multipotent (Jackson *et al.*, 2001; Krause *et al.*, 2001; Theise *et al.*, 2000; Vourc'h *et al.*, 2004). This interest in adult stem cells has in particular been triggered by the numerous ethical dilemmas surrounding the use of embryonic stem cells in preclinical and clinical research (Henningson *et al.*, 2003; McLaren, 2001). The best-characterized stem cell compartment is the bone marrow consisting of two stem cell populations, referred to as the hematopoietic and the mesenchymal

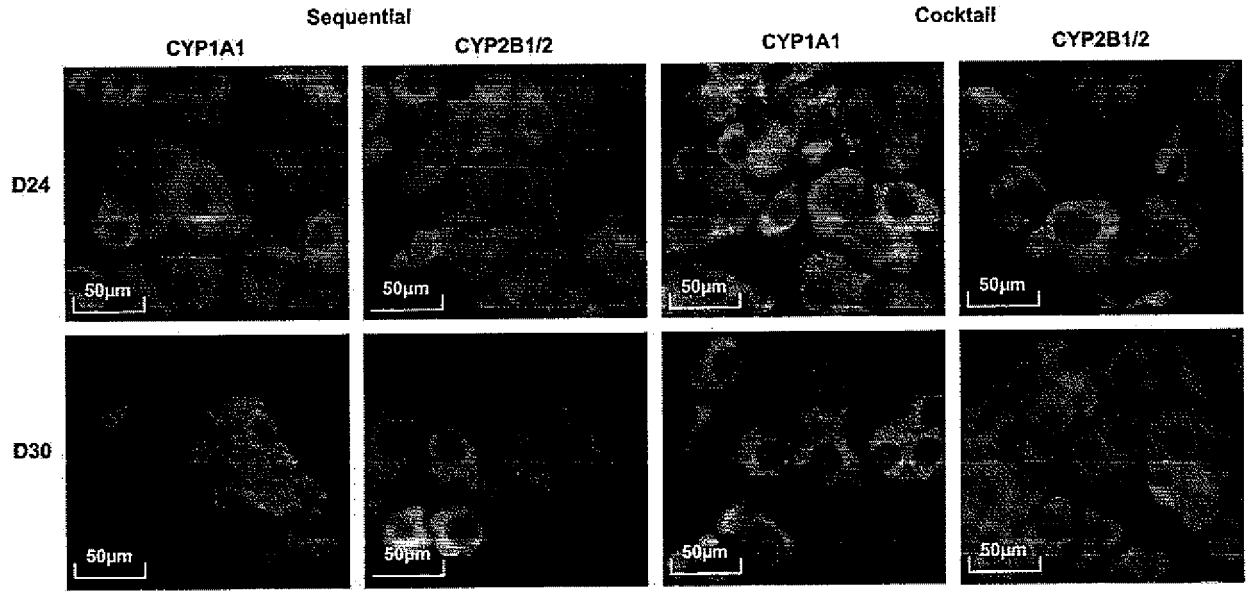

FIG. 9. Detailed view of expression of phase I biotransformation enzymes at days 24 and 30 upon sequential or simultaneous exposure to liver-specific factors. Immunocytochemistry was performed for CYP1A1-cy3 and CYP2B1/2-cy3. Mitochondria and endoplasmic reticulum were counterstained with a green fluorescent carbocyanine. Nuclei were counterstained with DAPI. Original magnification of ×320. Scale bar, 50 µm. Stainings shown have the same magnification and are representative for three separate experiments.

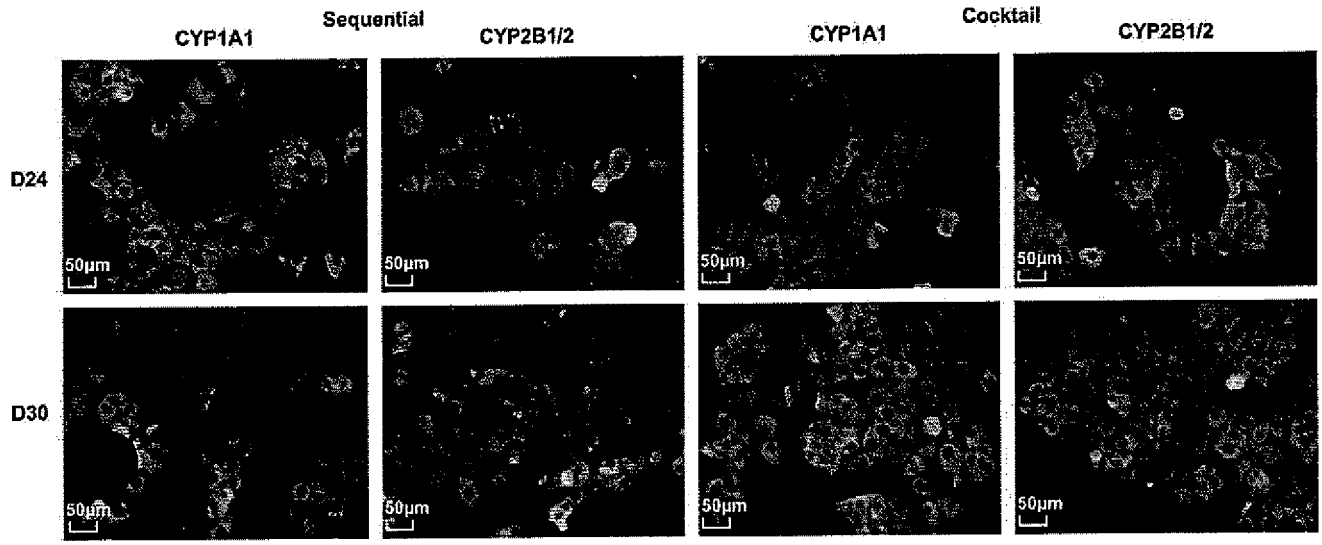

FIG. 10. Overall view of expression of phase I biotransformation enzymes at days 24 and 30 upon sequential or simultaneous exposure to liver-specific factors. Immunocytochemistry was performed for CYP1A1-cy3 and CYP2B1/2-cy3. Mitochondria and endoplasmic reticulum were counterstained with a green fluorescent carbocyanine. Nuclei were counterstained with DAPI. Magnification of ×112. Scale bar, 50 µm. Stainings shown have the same magnification and are representative for three separate experiments.

stem cells (Huttmann *et al.*, 2003). Previously, Schwartz *et al.* (2002) described a population of cells in postnatal rat bone marrow, copurified with mesenchymal stem cells, that were capable of differentiating into cells of endodermal (hepatocytes) origin upon exposure to well-defined hepatogenic factors. These culture conditions yielded, however, a mixture of epithelioid cells and other cell types. Therefore, attempts were made here to improve the hepatic differentiation process through exposure of BMSC to the same liver-specific factors in a sequential time-dependent manner, reflecting their secretion during *in vivo* hepatogenesis (Duncan, 2000; Jung *et al.*, 1999; Kinoshita and Miyajima, 2002; Schmidt *et al.*, 1995; Zaret, 2002).

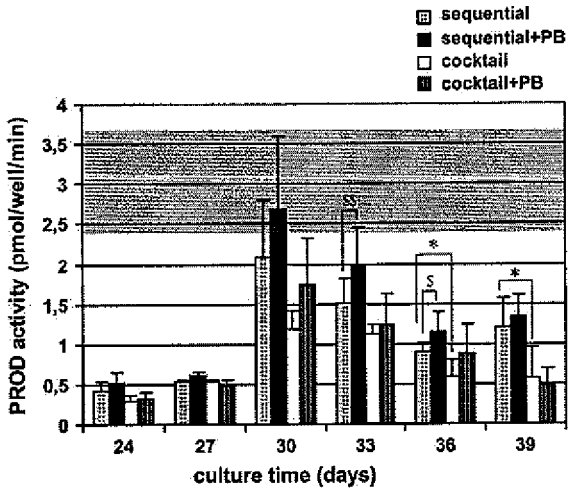

FIG. 11. PROD activities and responsiveness to 1mM PB in differentiated BMSC upon sequential or simultaneous exposure to liver-specific factors. PB was added daily, starting on day 24. Gray area represents PROD activity measured in untreated 4 h-cultured adult rat hepatocytes. The graph is representative for five separate experiments, each, performed in duplicate. *: PROD activity significantly differs among sequentially- and cocktail-exposed BMSC with $p < 0.05$ (Student's *t*-test). $, $$: PB significantly induced PROD activity of sequentially-exposed BMSC with $p < 0.05$ and $p < 0.01$, respectively (Student's *t*-test).

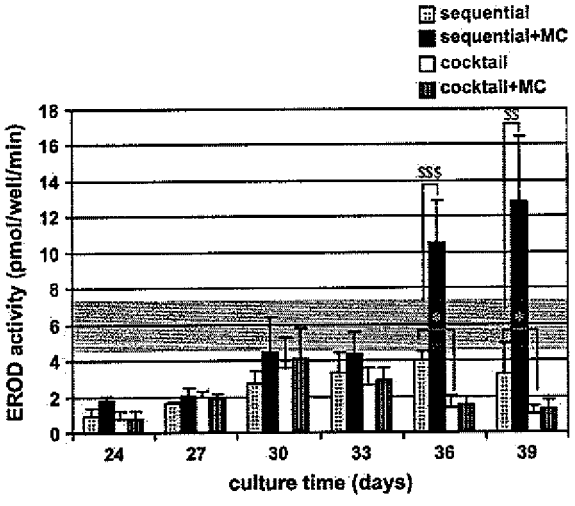

FIG. 12. EROD activities and responsiveness to 2µM MC in differentiated BMSC upon sequential or simultaneous exposure to liver-specific factors. MC was added daily, starting on day 24. Gray area represents EROD activity measured in untreated 4 h-cultured adult rat hepatocytes. The graph is representative for five separate experiments, each performed in duplicate. *: EROD activity significantly differs among sequentially- and cocktail-exposed BMSC with $p < 0.05$ (Student's *t*-test). $$, $$$: MC significantly induced EROD activity of sequentially exposed BMSC with $p < 0.01$ and $p < 0.001$, respectively (Student's *t*-test).

Under these culture conditions, BMSC acquired morphological features (polygonal-shaped and binucleated cells) similar to those of primary hepatocytes (Ferrini *et al.*, 1997; Katsura *et al.*, 2002). Furthermore, more than 85% of these epithelioid cells expressed liver-associated genes and proteins (AFP, HNF3β, ALB, CK18, and HNF1α) in a comparable time-dependent manner as observed during *in vivo* liver embryogenesis. Indeed, AFP expression is first detected in embryonic endoderm around E8.5 (Cascio and Zaret, 1991) and precedes ALB and HNF1α expression, detected around E9.5 and E10.5, respectively (Ott *et al.*, 1991; Shiojiri, 1981). This finding implicates that, in this setup, the BMSC differentiation process could serve as a model of early mammalian endoderm differentiation. In contrast, upon exposure to a cytokine/growth factors-cocktail, the expression patterns differed from the normal sequence seen during *in vivo* hepatogenesis as HNF1α expression preceded that of ALB. Indeed, HNF1α is only expressed in fully differentiated cells and not in un- or dedifferentiated cells (Cereghini *et al.*, 1988), as was noticed here upon cocktail treatment. In addition, significantly lower levels of liver-specific markers were expressed. The higher levels of ALB and CK18 expression in the sequential condition are probably due to the higher levels of both the early (HNF3β) and late (HNF1α) transcription factors. It is well documented that liver-enriched transcription factors act cooperatively and synergistically to promote liver-specific gene transcription (Cereghini *et al.*, 1992; Darlington, 1999; Duncan, 2000; Hayashi *et al.*, 1999; Shim *et al.*, 1988). In this regard, it was previously shown that HNF3β positively regulates the expression of HNF4α and HNF1α (Darlington, 1999; Duncan *et al.*, 1998). Furthermore, it is believed that HNF3β serves as the initiator of a cascade of regulatory events resulting in endoderm induction (Ang *et al.*, 1993; Darlington, 1999; Duncan, 2000; Levinson-Dushnik and Benvenisty, 1997). Hence, the minor changes in HNF3β expression levels in the cocktail condition may only result in low levels of ALB and CK18 transcripts and protein.

The initiation and induction of AFP expression is not yet completely understood. It can be assumed that additional factors are involved in its transcriptional activation, as in both culture conditions, only minimal levels of HNF3β were detected at the time of AFP expression. Further research will be needed to fully elucidate the transcriptional hierarchy mediating differentiation of BMSC toward hepatocytes.

The presence of both morphologic and phenotypic features, similar to that of primary hepatocytes, does, however, not fully prove the differentiation of BMSC into mature hepatocytes. Indeed, during the terminal step of liver organogenesis, the liver becomes a functional and metabolic organ, performing an essential role as detoxifying center of the body (Gomez-Lechon *et al.*, 2004; Kinoshita and Miyajima, 2002; Zaret, 2002). Interestingly, functional maturation occurred in both experimental setups, but to a different extent. Hepatic metabolic functions, including ALB secretion, urea production, storage of glycogen, and CYP-activity/inducibility, were manifested most prominently upon sequential exposure to hepatogenic factors. Under these culture conditions, ALB secretion was in fact significantly upregulated to levels comparable to those obtained in both 2- to 7-day–old immobilization and 7-day–old monolayer cultures of primary rat hepatocytes. The latter measurements are performed on a regular basis in our laboratory (Beken *et al.*, 2001; Vanhaecke *et al.*, 2004). In addition, both the urea production and EROD/PROD activities reached levels comparable to 4 h-cultured primary rat hepatocytes. Response to prototype inducers was as expected: pronounced upon exposure to MC and discrete upon PB treatment. The level of induction, however, remained lower in comparison to cultured adult rat hepatocytes. More specifically, EROD activity increased up to fourfold after 15-day exposure to MC in sequentially exposed BMSC versus maximal sevenfold in 2-day treated rat hepatocytes (Donato *et al.*, 1993). Nevertheless, to our best knowledge, this is the first time that EROD (CYP1A1/2) activity/inducibility is demonstrated in hepatocyte-like cells derived from BMSC.

The less mature phenotype of cocktail-exposed cells could possibly be ascribed to altered and lower expression of HNF-type liver-enriched transcription factors in this setup. Experiments using hepatoma cell lines and HNF-null mice have in fact demonstrated the important role of HNFs in the regulation of genes that are involved in biotransformation (*Cyps*) and ammonia metabolism (ornithine-transcarbamylase gene) (Gomez-Lechon *et al.*, 2004; Inoue *et al.*, 2002; Rodriguez-Antona *et al.*, 2002). Similar to the results reported here, inducible CYP2B1/2-activity was also found by Schwartz *et al.* (2002) after exposure to a cocktail of the same cytokines and growth factors, although at an earlier time in culture. Some variation in time-specific gene and protein expression could probably be attributated to intraspecies differences and subtle changes in the differentiation procedure (i.e., type of culture plate coating, serum, etc.).

In summary, during the first 18 days of the hepatic differentiation process of BMSC, cells, and sequentially exposed BMSC in specific, underwent a sequential array of developmental stages, characterized by the down- and upregulation of early and late liver-specific markers, respectively. As differentiation progressed, i.e., from day 18 onward, expression of mature hepatic markers persisted at steady levels (data not shown) and cells gradually underwent functional hepatic maturation. In specific, sequentially treated BMSC accomplished hepatic functions at levels comparable to those of primary rat hepatocytes, cultured for 4 h to 2 days. Our results thus clearly show that a more pronounced and homogeneous differentiation of BMSC into functional hepatocyte-like cells can be obtained by sequentially directing the differentiation process analogous to liver embryogenesis. Moreover, differentiation appears to occur via steps commonly defined for *in vivo* endodermal lineage specification and subsequent hepatocyte differentiation and maturation. Further investigations, in order

to elucidate the molecular mechanisms underlying the changes described herein, are underway.

This model opens new perspectives: it may not only be applicable to study endoderm differentiation *in vitro* but it also offers the possibility to purify and culture multipotent stem cells from nonembryonic origin as an unlimited cell source for pharmaco-toxicological research and testing, and cell and organ development. It might even open a road to trigger cell fate and "trans"-differentiate uncommitted cells from different tissues towards endodermal lineages.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

ACKNOWLEDGMENTS

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